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(54) Title: DE-MYRISTOLATED LIPOPOLYSACCHARIDE OF GRAM-NEGATIVE BACTERIA

(57) Abstract

A method of making gram-negative bacteria by inhibiting synthesis or activity of the protein encoded by *lpxF* (*msbB*). The gram-negative bacteria of the invention lack the lipid A myristic acid moiety and are hosts for production of immunogens or heterologous proteins. The de-myristolated LPS may be used as an LPS antagonist of LPS-mediated activation or as an adjuvant.

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DE-MYRISTOLATED LIPOPOLYSACCHARIDE OF GRAM-NEGATIVE BACTERIA

BACKGROUND OF THE INVENTION

The lipopolysaccharide (LPS) of gram-negative bacteria can act as a potent stimulator of inflammation even at sub-nanogram quantities. Stimulation occurs primarily through the LPS receptor, CD14. Wright et al., Science, 249:1431-1433 (1990). This receptor is present in a membrane bound (mCD14) and soluble (sCD14) form, each of which defines a pathway of cellular activation.

Monocytes and neutrophils contain mCD14 on their surface and produce several cytokines in response to the presentation of LPS via a serum protein designated lipopolysaccharide binding protein (LBP). Wright et al., Id.; Couturier et al., J. Immunol., 147:1899-1904 (1991). The secretion of these cytokines (e.g., tumor necrosis factor and interleukin-1 by macrophages) elicits a cascade of cytokine production by other cells of the immune system which, in turn, is associated with the symptomology of LPS intoxication, namely, leukocytosis, shock, disseminated intravascular coagulation, and death. Beutler et al., Annu. Rev. Immunol., 7:625-655 (1989). When the cytokines initially produced by monocytes exert their effect on other cells in the inflammatory cascade, this resultant mCD14 dependent pathway has been called indirect activation, since the cascade is not directly activated by LPS.

Direct activation of non-myeloid cells such as epithelial and endothelial cells by LPS requires sCD14. Pugin et al., Proc. Natl. Acad. Sci. USA, 90:2744-2748 (1993). The sCD14-LPS complex is believed to interact with a membrane receptor for cellular activation. One result of either direct or indirect activation of endothelial cells by LPS is the expression of E-selectin, an adhesion molecule known to be required for leukocyte exit from the vascular compartment. Bevilacqua et al., Proc. Natl. Acad. Sci USA, 84:9238-9242 (1987); Springer, Cell 76:301-314 (1994).

Studies have demonstrated that the lipid A moiety is the primary component of LPS responsible for host cell activation. Data obtained from both partial enzymatic degradation of lipid A and chemical synthesis of lipid A analogues has elucidated many of the structural requirements for monocyte activation. Takada and Kotani, Molecular Biochemistry and Cellular Biology, eds. Morrison and Ryan, pp. 107-130 (1992); Pohlman et al., J. Exp. Med. 165:1393-1402 (1987). Acyloxyacyl groups (i.e., myristate and laurate fatty acids) have been implicated as components of lipid A that play a critical role in its stimulatory ability.

In pathogens that have been associated with chronic infectious disease, such as *Porphyromonas gingivalis* or *Helicobacter pylori*, native forms of LPS either lack or have altered forms of the acyloxyacyl "piggyback" fatty acids normally found in *E. coli* and *S. typhimurium*. Dalla Venezia et al., Eur J. Biochem., 151:399-404 (1985); Ogawa, T., FEBS, 332:197-201 (1993). Highly purified LPS from these bacteria has been isolated and shown to lack the ability to stimulate endothelial cells to express E-selectin. Darveau et al., Infect. Immun. 63:1311-1317 (1995). Indeed, LPS from *P. gingivalis* was also able to block the stimulatory ability of *E. coli* LPS. Further, enzymatically deacylated *S. typhimurium* LPS does not stimulate neutrophil adhesion (Pohlman et al., J. Exp. Med. 165:1393-1402 (1987)) and presumably is similar to the LPS of bacteria associated with chronic infections in failing to elicit the presentation of E-selectin on endothelial cells. Additionally, lipid A antagonists, such as analogs of *Rhodobacter sphaeroides* have been reported to block LPS activation of macrophages. European Patent No. 536969; Golenbock et al., J. Biol. Chem., 266:19490-19498 (1991); and Takayama et al., J. Biol. Chem., 258:7379-7385 (1983).

In *E. coli*, mutations have been isolated at most steps of the lipid A biosynthetic pathway beginning at the first committed step in lipid A biosynthesis, UDP-*N*-acetylglucosamine (UDP-GlcNAc) acyltransferase (encoded by the *lpxA* gene), to the formation of KDO_2-Lipid IV_A. Raetz, J. Bacteriol., 175:5745-5753 (1993)). However, only temperature

sensitive mutations have been identified in the this pathway, indicating that synthesis of lipid A is essential for cell survival. Mutations have been identified that affect the cell wall fatty acid composition. Karow et al., J. Bacteriol., 174:7407-7418 (1992). In that study, it was also reported that inactivation of *msbB*, a multicopy suppressor of the temperature-sensitive phenotype of *htrB*⁻ mutants, reduced but did not eliminate levels of LPS myristic acid.

The possibility of LPS endotoxemia complicates the preparation and administration of proteins which have been produced in a bacterial expression system. Nevertheless, bacterial expression systems may still be advantageous due to their association with short generation times, large yields, and low costs. Certain compositions can only be expressed in bacteria due to their toxicity in eukaryotic hosts. Furthermore, whole bacteria are often administered in vaccine preparations. For example, *E. coli* has also been used to produce recombinant vaccines, such as the major antigen (VP1) of the foot-and-mouth disease virus.

Due to its extreme toxicity, LPS contamination is to be scrupulously avoided in pharmaceutical preparations and its effects *in vivo* must be rapidly and efficiently inhibited. It stands to reason that methods of reducing the risk of endotoxin contamination, or at least toxicity associated with endotoxins, as well as methods of treating LPS endotoxicity are needed and would have great utility in the bioprocessing and biomedical fields. Quite surprisingly, the present invention fulfills these needs and provides other related advantages.

SUMMARY OF THE INVENTION

The present invention provides compositions and methods of using gram-negative bacteria, or products thereof, having a form of LPS deficient in levels of the myristic acid moiety. De-myristolated LPS (dmLPS) is substantially less able to activate the inflammatory cascade than wild-type LPS. De-myristolated gram-negative bacteria are particularly useful in bacterial production processes where LPS contamination must

be avoided. Further, de-myristolated LPS itself may be used as an LPS-mediated inflammation antagonist, or adjuvant.

In one aspect, the present invention relates to compositions, and a method of making, heterologous peptides, proteins or other heterologous macromolecules in a de-myristolated lipid A gram-negative bacterial culture. The method comprises culturing gram-negative bacteria in which synthesis or activity of *lpxF* is inhibited and therein expressing a gene coding for the heterologous peptide, polypeptide or protein (hereinafter collectively referred to as "protein"). In one embodiment, synthesis is inhibited by preventing transcription or translation of *lpxF* nucleic acids. In another embodiment, *lpxF* is inhibited as a result of a promoter, operator or structural gene mutation. The *lpxF* genes of the gram-negative bacteria are typically at least 50% homologous to the *E. coli lpxF* gene or a fragment thereof of at least 15-25 nucleotides in length. The heterologous proteins may be viral, eukaryotic or of a different bacterial species or serotype.

In another aspect, the present invention relates to compositions and methods of making de-myristolated gram-negative bacteria or biosynthesized (or catabolized) products thereof for use in compositions comprising a pharmaceutically acceptable carrier, e.g., for vaccines and adjuvants. The method comprises culturing gram-negative bacteria in which synthesis or activity of *lpxF* is inhibited in said bacteria and suspending the bacteria or products thereof in a pharmaceutically acceptable carrier. In one embodiment, the gram-negative bacteria are selected from the group comprising *Salmonella typhimurium, Vibrio cholerae, Bordetella pertussis, and Haemophilus influenzae*. In another embodiment, the gram-negative bacteria comprise a gene coding for an immunogenic protein. The immunogenic protein may be prokaryotic, eukaryotic or viral and may be secreted into the media, periplasmic space, or inserted into the outer membrane. In a further embodiment, the bacterial product is dmLPS for use as an immunological adjuvant. The dmLPS may be purified prior to use and can be conveniently derived from *E. coli*.

The present invention also relates to a method to antagonize the LPS-mediated inflammatory cascade by administration of a therapeutically effective dose of dmLPS. In another embodiment, the dmLPS is linked to a therapeutic or diagnostic composition such as an antibody or antibody fragment, protein, peptide, radioisotope, hormone, enzyme inhibitor, enzyme, sugar, or nucleic acid. In a further embodiment, dmLPS is linked to antibodies or fragments thereof capable of immunospecifically binding to TNF-α or IL-1.

DESCRIPTION OF THE SPECIFIC EMBODIMENTS

The present invention provides methods of producing, from or in gram-negative bacteria, immunogens, or foreign peptides, proteins or saccharides, in which the lipid A of the bacterial lipopolysaccharide (LPS) molecule lacks or is deficient in the myristic acid moiety and, consequently, is of substantially reduced endotoxicity. In another aspect, the present invention relates to methods of biosynthesizing de-myristolated LPS (dmLPS) for use as immunological adjuvants or endotoxin antagonists.

The methods of the present invention comprise inhibiting in gram-negative bacteria the formation or activity of the enzyme corresponding in function to the enzyme encoded by the *lpxF* (alternatively, "*msbB*") gene in *E. coli* (GenBank Accession No. M77039) (Karow et al., J. Bacteriol., 174:702-710 (1992). In *E. coli*, inactivation of the *lpxF* gene or gene product results in a de-myristolated form of lipopolysaccharide. Unless otherwise noted, LPS, as used herein, refers to the KDO_2-lipid A precursor as well as subsequent glycosylated forms. See, Raetz, J. Bacteriol., 175(18):5745-5753 (1993), incorporated herein by reference. KDO_2-lipid A is formed following attachment of two KDO residues to lipid IV_A and acylation with laurate and myristate residues.

The term "*lpxF*" refers to both the *msbB* gene of *E. coli* as well as its functional homologs in other gram-negatives as determined by at least one of three criteria: 1) the ability of the putative *lpxF* gene to restore the

myristolated-LPS phenotype upon expression in *lpxF* dmLPS *E. coli* or a gram-negative organism; 2) the ability to convert the LPS phenotype of a gram-negative from a myristolated to a de-myristolated form after inactivation of the putative *lpxF* gene; or 3) upon expression of the *E. coli lpxF* gene, the restoration of a myristolated-LPS phenotype in a gram-negative recipient having a dmLPS phenotype. Myristolated lipid A, the lipid component of LPS, is found in a wide variety of gram-negatives. Morrison and Ryan, eds., Molecular Biochemistry and Cellular Biology, CRC Press, Vol. 1, (1992); Raetz, Annu. Rev. Biochem., 59:129-170 (1990). The presence or absence of LPS can be assayed for by methods well known to those of skill in the art, e.g., activation of macrophages, GC or HPLC analysis of LPS fatty acids, or E-selectin expression by endothelial cells.

The *E. coli lpxF* gene, or fragments thereof, have at least about 50% nucleotide sequence homology to the *lpxF*, or subsequence thereof, of non-*E. coli* gram-negatives, more preferably 60% to 70%, and most preferably at least about 75%. The percentage of homology is calculated by comparing one sequence to another when aligned to corresponding portions of the reference sequence. Homology, as herein defined, is assessed without regard to insertions (i.e., spaces) or deletions (i.e., gaps) of nucleotides. The fragments are generally of the minimal size needed to ensure hybridization specificity in cDNA library screening. Typically, the fragments are at least 15-25 nucleotides in length, and generally comprise at least one, sometimes two, three or more, subsequences of at least 3, and sometimes 4, 5, 6, 7, 8, 9 or more, contiguous nucleotides from the *E. coli* lpxF gene. The presence in gram-negatives of a *lpxF* gene homologous to *E. coli lpxF* or fragment thereof is readily determined by methods well known to the skilled artisan. Nucleic acid sequence databases, such as GenEMBL, may be used in sequence homology searches to determine the presence of homologous genes in other gram-negative species or to identify and construct probes to conserved regions of *lpxF* sequences for use as probes. Further, techniques such as screening DNA libraries

with labeled oligonucleotide probes from *E. coli lpxF* may be used. Alternatively, expression libraries can be screened using polysera or monoclonals to the *E. coli lpxF* protein.

Conveniently, an oligonucleotide probe derived from *E. coli lpxF* is used to screen genomic DNA libraries of other gram-negative bacteria. Randomly labeled fragments of *lpxF* may be used; preferably, conserved regions of *lpxF* identified by alignment techniques from multiple gram-negative species are used to screen the libraries. Putative *lpxF* DNAs are cloned and expressed in *lpxF⁻ E. coli* in accordance with conventional molecular biology techniques as described in, e.g., Sambrook et al., Molecular Cloning, A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989. For example, using a functional assay, the putative *lpxF* gene is expressed in dmLPS *lpxF⁻ E. coli*. This mutant is assayed for conversion to a LPS phenotype, conveniently by activation of macrophages or endothelial cells. Activation can be determined by assaying, e.g., IL-1 or TNF-α secretion from macrophages, or E-selectin expression by endothelial cells. Binding of a LPS/LPS binding protein complex to CD14 receptors found on macrophages triggers macrophage activation and results in the production of the cytokines tumor necrosis factor-α (TNF-α) and interleukin 1 (IL-1), as described in e.g., Lee et al., J. Exp. Med. 175:1697-1705 (1992); Raetz et al., FASEB J. 5:2652-2660 (1991); Tobias et al., J. Biol. Chem. 264:10867-10871 (1989). Binding of LPS to endothelial cells activates expression of E-selectin. Antibodies to TNF-α, IL-1, and E-selectin are widely available from commercial sources, e.g., Pierce (Rockford, IL), R & D Systems (Minneapolis, MN).

Bacterial taxonomic relationships based on sequence similarities (e.g., rRNA homology studies) can be used to identify and isolate *lpxF* from other gram-negative bacteria of interest. In this method, *lpxF* from *E. coli* is used to identify and isolate *lpxF* from a species intermediate in phylogenetic distance between *E. coli* and the gram-negative bacteria of interest. The intermediate species is used to identify and isolate *lpxF* from a species more closely related

to the gram-negative of interest. This stepwise method can be used to bridge differences in *lpxF* sequence that exist between *E. coli* and other gram-negative bacteria.

Inhibition of *lpxF* synthesis may be achieved by interfering with transcription or translation of the *lpxF* gene product to reduce or prevent synthesis of the gene product. Alternatively, the activity of the *lpxF* gene product may be inhibited. For example, mutagenesis to prevent transcription may proceed by the use of alkylating agents to inactivate genes by the formation of alkylating agent-DNA adducts as with such reactive electrophiles as epoxides or nitrogen mustards, or photoreactive compounds such as psoralen. Prevention of transcription may also be achieved by mutagenesis of the structural gene, or operator or promoter regions. See, e.g., Watson et al., Molecular Biology of the Gene, 4th Ed., pp. 231-233, Benjamin/Cummings Publishing Co., Menlo Park, CA (1987). Due to phenotypic lag, *lpxF* is typically inhibited for a sufficient number of doubling periods prior to use of dmLPS bacterial strains to reduce LPS to an acceptable or desired level.

Translation may be prevented by the use of ribozymes (catalytic RNA) to cleave messenger RNA transcripts or by the use of anti-sense agents to form homo- or hetero-duplexed messenger RNA. Such translational inhibitors may be encoded by genes introduced into the targeted gram-negative bacteria. Alternatively, the activity of gene products may be inhibited by the use of e.g., enzyme inhibitors or by the creation of missense or nonsense mutations within the structural gene. As will be understood by the skilled artisan, "structural gene" refers to the DNA sequence from which messenger RNA is transcribed and typically comprises a 5′ leader for ribosome binding, a region or regions translated into peptides or proteins, and a 3′ stop codon.

Mutagenesis may by accomplished by such well known techniques as, e.g., chemical or radiological mutagenesis, by insertional mutagenesis via insertion sequences or transposable elements, or via recombination with a mutagenized copy of the gene. The mutations induced may be multi-basepair

lesions such as insertions or deletions, or point mutations such as additions or substitutions. Alternatively, mutagenesis may occur as a result of normal replicative errors or any combination of the above. The methods of the present invention are not limited to any particular means of inhibition of synthesis or activity and may proceed by any number of molecular biology or biochemical techniques. Inhibition of *lpxF* may be achieved *ex vivo* or *in vivo*. Thus, *lpxF* inhibition further provides a means to assay for *lpxF* inhibiting pharmaceutical compounds (e.g., enzyme inhibitors) for *in vivo* application (e.g., anti-infectives). Inhibition of synthesis or activity may be used to produce dmLPS levels up to greater than 50%, preferably at least 80% to 90%, more preferably from 95% to 100% of total LPS (mLPS + dmLPS). To identify inhibitors of the *lpxF*-encoded enzyme as a basis for antibiotic therapy, the wild-type enzyme is employed to screen for inhibitors. dmLPS is also used in a screening assay as a substrate for the *lpxF*-encoded enzyme to identify inhibitors of the reaction and thus anti-bacterial or other pharmaceutically useful agents. In another aspect organisms having a dmLPS phenotype, i.e., which do not express a fully functional *lpxF* gene, are used to provide an avirulent or less virulent host organism in which other factors, such as potential virulence factors can be tested. *lpxF* synthesis can be inhibited in a selected host organism which already expresses *lpxF* by techniques described herein, e.g., using homologous recombination, etc., to replace *lpxF* with a mutated *lpxF* gene to provide a dmLPS host organism against which or in which the effect of the factor of interest is determined.

The dmLPS bacteria formed by inhibition of *lpxF* may be used to produce immunogens for use in vaccines, to produce heterologous proteins, nucleic acids or saccharides, to produce dmLPS endotoxin antagonists, or to produce dmLPS for use as an immunological adjuvant. The genes encoding immunogens or heterologous macromolecules may be expressed prior to, during, or after inhibition of *lpxF* as desired. Exemplary immunogens for use as vaccines that are expressed from dmLPS bacteria according to the present invention are

derived from eukaryotes, such as eukaryotic cells (e.g., tumor antigens), protozoans or parasitic worms (e.g., *Trypanosoma*, *Schistosoma* or *Plasmodium*), bacteria (e.g., *Salmonella typhimurium*, *Vibrio cholerae*, *Bordetella pertussis*, and *Haemophilus influenzae*), or viruses such as those giving rise to influenza, measles, mumps, rubella, polio, rabies, yellow fever, AIDS, human papilloma virus, Epstein-Barr, herpes simplex 1 or 2, varicella, hepatitis B or C, etc. Immunogens may be substantially purified or introduced along with their dmLPS host. When purified, the immunogens for use as vaccines are preferably in inclusion bodies, secreted into the periplasmic space or secreted into the medium, or a fusion with such proteins. When the bacteria are given as a vaccine (i.e., a cellular vaccine), the immunogens are preferably an outer membrane protein or a fusion with such proteins. Thus, dmLPS bacteria may themselves be used as a vaccine, for production of foreign antigens, or both. Gram-negative bacteria that may be used as cellular bacterial vaccines include, e.g., *Salmonella typhimurium*, *Vibrio cholerae*, *Bordetella pertussis*, and *Haemophilus influenzae*, too name a few.

The dmLPS bacteria may also be used to produce heterologous proteins, saccharides, dmLPS, or other products to act as immunogens, or for diagnostic, therapeutic or industrial (e.g., catalytic) applications. Heterologous refers to those biosynthesized products (including proteins, polypeptides and peptides) which are not indigenous to the bacterial species producing them. The heterologous proteins produced according to the invention may be cytoplasmic, preferably periplasmic, or secreted into the media. The heterologous proteins may be from eukaryotes, eukaryotic viruses, or from prokaryotes or bacteriophage. The heterologous proteins may also be fused to native (i.e. indigenous) or heterologous proteins. Methods for production of foreign proteins in gram-negative bacteria are known to the skilled artisan. See, e.g., Sambrook, supra. Conveniently, *E. coli* expression systems may be employed. Alternatively, other gram-negative expression systems may be used. For example,

various vectors have been developed for cloning in *Pseudomonas putida*. Franklin et al., Proc. Natl. Acad. Sci. USA, 78:7458-7462 (1981).

The dmLPS of the present invention can be used for pharmaceutical compositions, particularly for administration to mammals such as livestock and other veterinary applications, and to humans. Thus, dmLPS may be used as an antagonist to the activation of LPS-mediated inflammation. For use as an antagonist, sufficient dmLPS is administered such that the cells involved in LPS-mediated inflammation are not substantially activated; preferably, the level of activation, as measured by E-selectin expression by human umbilical vein endothelial cells or TNF-α production by adherent monocytes, is inhibited to 25% or less of the unantagonized values. In one embodiment, LPS-mediated activation is antagonized by binding of dmLPS to an LPS specific receptor, such as CD14 or LPS binding protein (LBP). In another embodiment, the LPS antagonism can be displayed in the activation pathway subsequent to the CD14/LPS binding protein complex formation.

De-myristolated LPS derived from LpxF$^-$ gram negative bacteria as described herein can also be used in a pharmaceutical preparation as adjuvant to improve the immunogenicity of antigens. The dmLPS adjuvant can be provided in a range of molecular weights, from a single monomer of de-myristolated KDO_2-Lipid A to more mature, higher molecular weight forms of dmLPS, with or without the O-antigen repeat or outer or inner cores. The dmLPS adjuvant is typically administered in a pharmaceutically acceptable carrier such as, but not limited to, a saline solution, phosphate buffered saline (PBS), as a water or buffer emulsion, or in a liposomal formulation. Generally, for use as an adjuvant, dmLPS is administered from about 25 to 500 mg for a 70 kg individual. The adjuvant may be provided simultaneously, prior to, or following administration of the antigen per the direction of the clinician.

In therapeutic applications, compositions are administered to a mammal, including humans, in an amount

sufficient to at least partially arrest symptoms and/or complications. This amount is defined as a "therapeutically effective dose." Amounts effective for this use will depend on, e.g., the dmLPS composition, the manner of administration, the stage and severity of the LPS intoxication being treated, the weight and general state of health of the patient, and the judgment of the prescribing clinician. Generally, the range for use as an antagonist to an LPS-mediated inflammatory reaction, the therapeutic composition is from about 1 mg dmLPS to about 750 mg for a 70 kg patient. It must be kept in mind that the methods of the present invention may be employed in serious disease states, that is, life-threatening or potentially life threatening situations. In such cases, in view of relatively nontoxic nature of dmLPS, it is possible and may be felt desirable by the treating clinician to administer substantial excesses of dmLPS, alone or as a linked composition. For use as an antgonist, administration of dmLPS may begin prophylactically, at the first sign of endotoxemia, and continuing until symptoms of endotoxemia have abated, per the discretion of the clinician. Preferably, for therapeutic or pharmaceutical use the dmLPS is purified from the bacterial species by a number of methods known to the skilled artisan. See, e.g., Ames, J. Bacteriol., 95:833-843. The dmLPS is generally purified from bacterial membranes, preferably purified from bacterial membrane proteins, and more preferably at least 75% to 95% (wt/wt) pure. The dmLPS may also be hydrolyzed from core sugars, including the two KDO residues, to form an LPS composition with a lipid A component having the structure corresponding to $\beta(1,6)$ D-glucosamine disaccharide 1, 4' bisphosphate with the following lipid placement: 2-[14:0 (3-OH)], 3-[14:0 (3-OH)], 2'-[14:0 (3-O-12:0)], 3'-[14:0 (3-OH)], using the ring numbering of Galanos et al., Eur. J. Biochem., 148:1-5 (1985), incorporated herein by reference.

De-myristolated LPS, vaccines, immunogens, proteins or other pharmaceutically useful compositions of the present invention may be administered parenterally, e.g., intravenously, subcutaneously, intradermally, or intramuscularly. Thus, the invention provides compositions

for parenteral administration which comprise a solution of the dmLPS, vaccines, immunogens, and/or proteins suspended (or otherwise dissolved) in a pharmaceutically acceptable carrier, often with a surfactant. The surfactant must, of course, be nontoxic. Representative of such agents are the esters or partial esters of fatty acids, such as caproic, octanoic, lauric, palmitic, stearic, linoleic, linolenic, olesteric and oleic acids with an aliphatic polyhydric alcohol or its cyclic anhydride. Mixed esters, such as mixed or natural glycerides may be employed. These compositions may be sterilized by conventional, well known sterilization techniques, or may be sterile filtered. The resulting composition may be packaged for use as is, or lyophilized, the lyophilized preparation being combined with a sterile solution prior to administration. The compositions may contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions, such as pH adjusting and buffering agents, tonicity adjusting agents, wetting agents and the like, for example, sodium acetate, sodium lactate, sodium chloride, potassium chloride, calcium chloride, sorbitan monolaurate, triethanolamine oleate, etc.

The following examples are offered by way of illustration, not by way of limitation.

Example 1

This Example describes the generation of *E. coli* mutants having a reduced ability to stimulate the inflammatory process, as measured by ability to stimulate human endothelial cells to produce E-selectin.

E. coli strain JM83 *F' ara (lac-proAB)/rpsL* (Str^r) [80 d*lac(lacZ)M15]* was the K-12 strain selected for mutagenesis. Yanisch-Perron et al., Gene 33:103-119 (1985). JM83 was grown to late-log phase in LB broth (Sambrook, supra) supplemented with 0.2% maltose. The cells were isolated by centrifugation and suspended in 1/2 volume of 10 mM $MgSO_4, 7H_2O$, 10 mM Tris, pH 7.5. *E. coli* Q1 (deBruijn et al., Gene 27:131-149 (1984)) was used for propagation and titration of a λ467 phage stock carrying the transposon Tn5. Jorgensen et al.,

Molec. Gen. Genet. 177:65-72 (1979). Serial dilutions of the λ467 phage stock (7×10^{10} pfu/ml) were mixed with 0.1 ml aliquots of JM83 cells and the mixture incubated at room temperature for five minutes. Next, 0.9 ml of LB broth was added to the infected cells and the culture was incubated between 15 and 30 minutes at 37°C with shaking, to allow Tn5 transposition and expression of the kanamycin resistance marker. Cells from each culture were then concentrated by centrifugation, suspended in 0.1 ml of LB broth and spread onto LB agar plates containing kanamycin sulfate at a final concentration of 75 μg/ml.

An expression period of at least 15 min. following adsorption of the λ467 phage was necessary for isolation of kanamycin mutants. However, siblings could be identified with an expression period of ≥45 min. and therefore an expression period of 15-30 min. was used. After growth overnight at 37°C, Tn5 mutants were individually picked from plates infected at an MOI of <1 into 96-well, low protein binding, microwell plates (Corning Costar, Cambridge, MA) that contained 0.15 ml of LB broth, with kanamycin at a final concentration of 75 μg/ml. Tn5 mutants were picked only from plates where the multiplicity of infection (MOI) was ≤1 to reduce the possibility of isolating double mutants. Following overnight growth at 37°C, without shaking, the microtiter plates were centrifuged to pellet the cells. The cells were resuspended in fresh LB broth containing 15% glycerol, then the plates were frozen on dry ice and stored at -70°C.

The bacterial mutants were then screened for reduced ability to stimulate endothelial cells to produce E-selectin. 5704 individual Tn5 mutagenized isolates of *E. coli* JM83 were screened against human umbilical vein endothelial cells (HUVEC) using an E-selectin stimulation ELISA assay. Darveau et al., Infect. Immun. 63:1311-1317 (1995), incorporated herein by reference. HUVEC cells were obtained from Clonetics (San Diego, CA) and were propagated in HUVEC growth media. Id. E-selectin stimulation assays used cells passed no more than four times. Stimulation assays were carried out in the presence of 5% pooled normal human serum (Gemini Bioproducts,

Calabasas, CA), or in 5% human albumin (Immuno-US Inc., Rochester, MI) when serum free conditions were desired. An anti-E-selectin antibody, BBA1, was purchased from R and D Systems (Minneapolis, MN), and an $F(ab')_2$ goat anti-mouse IgG-specific HRP-conjugated second step detection antibody was purchased from Jackson Immunoresearch Labs (West Grove, PA). Non-mutagenized JM83 cells grown in microwell plates were used to determine the appropriate dilutions necessary to observe serum dependent stimulation of E-selectin on HUVEC cells. Dilutions of cells in stimulation media (Darveau et al., supra) without serum demonstrated serum-independent stimulation at high cell numbers. Thus, a dilution that gave an absorption value in the E-selectin assay (Darveau et al., supra) of between 0.4 and 0.8 in the presence of 5% normal human serum, but a serum-free value equal to background, was used. Each microwell plate contained controls consisting of non-stimulated HUVEC cells, wells stimulated with TNF-α, and wells stimulated with the non-mutagenized JM83 strain.

From the initial screen, 29 prospective mutants were identified and were then subjected to a secondary screen. This consisted of growing cultures of prospective mutants and the JM83 control overnight in LB broth at 37°C with shaking. The culture densities were measured and adjusted using LB broth to an A_{660} value equal to 1.0 (4×10^8 cfu/ml). Serial 10-fold dilutions of the cultures were then made in stimulation media and the samples placed onto HUVEC cells to measure stimulation of E-selectin. This secondary screening identified the Tn5 mutant designated BMS67C12 as having significant loss in the ability to stimulate E-selectin formation on HUVEC cells. Whole cells of the *E. coli* strain BMS67C12 have an approximately 1 to 2 log reduction in their ability to stimulate HUVEC cells to produce E-selectin when compared to the parental strain JM83.

Example 2

This Example demonstrates the isolation of LPS from the mutant BMS67C12 and analysis of LPS fatty acids.

For rapid purification of LPS and analysis of fatty acids, 10 mg of lyophilized cells were extracted three times with 45% phenol at 70°C and the cooled aqueous layers recovered by centrifugation. After extraction with diethyl ether, the aqueous phase was evaporated to dryness under a flow of nitrogen gas at 45°C. For large scale isolation of high purity LPS, the phenol-water method of Westphal and Jann was used. Westphal et al., Methods in Carbohydrate Chemistry, ed. Whistler R.L., Academic Press, Inc., NY, pp. 83-91 (1965).

To determine whole cell phospholipid composition, 50 mg aliquots of lyophilized cells were extracted with chloroform/methanol by the method of Hanson et al. (Manual of Methods for General Microbiology, ed. Gerhardt, P., pp. 328-364 (1981)) prior to derivation and analysis. For whole cell fatty acid analysis, 5 mg aliquots of lyophilized cells were derived and analyzed as described below.

LPS fatty acids, phospholipids, and whole cell fatty acids were derivatized to fatty acid methyl esters by methanolysis in 2M methanolic HCl at 90°C for 18 hr. with the addition of pentadecanoic acid as an internal standard. After the addition of an equal volume of saturated NaCl solution, the methyl esters were extracted with hexane and analyzed by gas chromatography with a 50m x 0.25mm HP-1 capillary column on a Hewlett-Packard 5890 gas chromatograph with a programmed temperature ramp from 90°C to 225°C. Limulus Amebocyte Lysate (LAL) testing was done using a kinetic assay in an automated microplate reader according to the manufacturers instructions (Endosafe, Charleston, SC).

Gas chromatography of highly purified LPS revealed that LPS isolated from mutant BMS67C12 was lacking the 14:0 myristoyl fatty acid moiety. A small amount of 14:0 that was seen in the chromatogram was probably due to phospholipid contamination as indicated by the presence of 16:0, which was also present in a less than stoichiometric amount. Both whole cell fatty acids and phospholipid content did not vary significantly compared to the parental strain. Thus, the analysis of whole cell fatty acids and phospholipids indicated that the lack of 14:0 observed in the LPS of the mutant

BMS67C12 was not due to an alteration in the overall cellular pool of fatty acid substrates needed for lipid A biosynthesis. Testing of the LPS in the LAL-assay gave values of 2.1×10^6 EU/mg for JM83 and 5.0×10^6 EU/mg for BMS67C12.

Highly purified LPS from each the mutant strain and JM83 was isolated and examined for their ability to stimulate HUVEC cells to produce E-selectin as described above. It was observed that the LPS from the BMS67C12 strain had lost most of its ability to stimulate E-selectin production.

Example 3

This Example describes the reduced ability of the BMS67C12 mutant lacking the 14:0 myristoyl fatty acid to stimulate adherent monocytes, as determined by production of TNF-α.

Adherent monocytes were isolated from the whole blood of individuals randomly selected from a population of normal human donors. Whole heparinized blood from an individual donor was diluted with one volume of RPMI 1640 medium (GIBCO/BRL, Gaithersburg, MD) and overlayered onto Lymphocyte Separation Medium (Organon Teknika Corp., Durham, NC). The gradient was centrifuged at 500 x g for 30 min. at room temperature. The lymphocyte layer was removed and the lymphocytes diluted with 1 vol. of RPMI 1640. The cells were pelleted and washed once more in RPMI 1640 then resuspended in a small volume of RPMI 1640 containing 5% fetal calf serum. Following counting and dilution to 5×10^6 cells/ml, 1 ml aliquots were added to each well of 24-well tissue culture plates. Cells were allowed to adhere for 1 hour at 37°C, then the monolayers were washed 3 times with serum free RPMI 1640. Typically, adherent cells represented 10% of the lymphocyte population. Following washing, RPMI 1640 medium containing 5% normal human serum and *E. coli* strains JM83 or BMS67C12, or LPS purified from JM83 or BMS67C12 was added. After 4 hours of stimulation, the culture supernatants were harvested and assayed for the presence of TNF-α using a human TNF-α specific ELISA assay (Amersham Corp., Arlington Heights, IL).

BMS67C12 whole bacterial cells with de-myristolated LPS (dmLPS) were approximately 3 logs less effective as the parental *E. coli* strain at stimulating adherent monocytes to produce TNF-α. When comparing purified LPS in this system, the effect was even more pronounced, with a 10,000-fold lower activity of dmLPS when compared to normal *E. coli* LPS (Table 1).

TABLE 1. LPS Induced E-selectin Expression and TNF-α Production

LPS (ng/ml)	E-SELECTIN (A_{450}/A_{630}) JM83	E-SELECTIN (A_{450}/A_{630}) BMS67C12	TNF-α (pg/ml) JM83	TNF-α (pg/ml) BMS67C12
10,000				1,850
1,000	0.796	0.2185		1,210
100	0.6555	0.2515		297
10	0.628	0.075		84
1	0.5955	0.013	2,540	41
0.1	0.269	0	2,050	40
0.01	0.038	0	930	33
0.001	0	0	217	0
0.0001	0	0	0	0

Example 4

This Example describes the role of the CD14 pathways in the LPS-mediated stimulation of adherent monocytes and endothelial cells.

LPS stimulation of endothelial cells has been proposed to occur via a soluble form of the CD14 receptor (sCD14). Pugin et al., Proc. Natl. Acad. Sci. USA, 90:2744-2748 (1993); Frey et al., J. Exp. Med., 176:1665-1671 (1992). In contrast, LPS stimulation of adherent monocytes (macrophages) and polymorphonuclear leukocytes is believed to occur via a membrane bound form of CD14 (mCD14). Wright et al., Science 249:1431-1433 (1990); Wright et al., J. Exp. Med., 173:1281-1286 (1991). As shown in Example 3, only relatively high doses of dmLPS were able to stimulate endothelial cells or adherent monocytes. To determine if the stimulation which was observed at the high doses required CD14, an anti-CD14 antibody (MY4) that has been shown to

specifically block stimulation at CD14 was used. Couturier et al., J. Immunol. 147:1899-1904 (1991); Lee et al., Proc. Natl. Acad. Sci. USA 90:9930-9934 (1993).

MY4 was added at varying concentrations to stimulation medium containing 5% normal human serum and incubated for 1 hour at 37°C. LPS (5 ng/ml) or dmLPS (500 ng/ml) was then added to the stimulation media and 100 µl aliquots of the mixtures were used in the HUVEC based E-selectin assay described above. To test the blocking of stimulation of adherent monocytes, isolated adherent monocytes were incubated with the MY4 antibody diluted in serum free stimulation medium for 1 hour at 37°C prior to adding of the LPS and 5% normal human serum. For adherent monocytes, LPS was used at 10 ng/ml and dmLPS was used at 100 ng/ml. After 4 hrs. of stimulation the level of TNF-α in the culture supernatants was determined. The MY4 antibody completely blocked both the high dose dmLPS stimulation of endothelial cells (as assayed by the expression of E-selectin) and the release of TNF-α when attempting to stimulate adherent monocytes. In contrast, whole cell stimulation in both assays was only partially blocked, suggesting that whole cells may contain non-CD14 dependent pathways for E-selectin or TNF-α activation. The ability of the MY4 antibody to block the ability of dmLPS to stimulate both endothelial cells and adherent monocytes, indicates that the stimulation seen at relatively high concentrations of dmLPS is still dependent on the CD14 receptor pathways and is not due to stimulation through an unknown alternative receptor pathway.

Example 5

This Example describes cloning and identification of the gene from BMS67C12 that contain a Tn5 insertion.

Total DNA was isolated from the *E. coli* strains JM83 and BMS67C12 using a modification of previously described methods. Somerville et al., J. Bacteriol., 156:168-176 (1983), incorporated herein by reference. Alternatively, one ml. of cells (approximately 1 X 10^9 cells) are pelleted and DNA

isolated using Easy-DNA (Invitrogen, San Diego, CA). DNA is resuspended, RNase treated, and extracted with a one-half volume of phenol/$CHCl_3$ (1:1). The aqueous phase is isolated and further extracted with a volume of $CHCl_3$. The DNA is ethanol/sodium acetate precipitated, washed with 80% ethanol, and resuspended in 100 µl of TE buffer. Samples of each DNA were digested with a variety of restriction enzymes known to lack sites in the Tn5 transposon. Restriction endonucleases and DNA modification enzymes were from commercial sources and used according to the manufacturer's instructions. For detecting Tn5 insertions, the plasmid pRZ102 was labeled using a digoxigenin random primed labeling and detection system (Boehringer Mannheim Corp., Indianapolis, IN). Plasmid pRZ102 carries the transposon Tn5 in a colE1 vector (Jorgensen et al., Molec. Gen. Genet., 177:65-72 (1979). Supercoiled plasmid DNA to be labeled for hybridization was first treated with an ATP-dependent DNase (Plasmid-Safe, Epicentre Technologies, Madison, WI) to eliminate any *E. coli* chromosomal DNA contamination. The samples were then electrophoresed in an agarose gel and the separated fragments blotted onto nitrocellulose using a modification of the Southern blotting technique (Sambrook et al., p. 7.37-7.52, supra), p 7.37-7.52 (1989)). It was found that both mutant strains contained single Tn5 insertions in their chromosomal DNA. In addition, single fragments that contained the Tn5 were identified with three of the restriction enzymes. *Kpn*I, *Eco*R1 and *Sac*I digests of BMS67C12 DNA were ligated into aliquots of the plasmid vector pUC18 (Yanisch-Perron et al., supra) that had also been digested with the same enzymes. These DNA libraries were transformed into *E. coli* strain JM109 (ibid.), then screened for clones that contained plasmids that were resistant to both ampicillin (100 µg/ml) and kanamycin (75 µg/ml).

Plasmids isolated from the *Sac*I libraries were identified and selected for further analysis. Plasmid pBMS67 with DNA from the *E. coli* BMS67C12 strain contained a genomic *Sac*I fragment of 8.2 kb. The single *Bam*HI site located in the center of Tn5 was used to subclone fragments on either side of

each Tn5 insertion site. An oligonucleotide primer 5'-ATGGAAGTCAGATCCTGG-3' [SEQ ID NO:1] targeted to the insertion sequence of the Tn5 (GenBank Accession No. L19386), was then used for directional dideoxy sequencing outward from the Tn5. This permitted the exact location of the Tn5 insertion to be identified in each clone and provided sufficient sequence information to search the GenEMBL database to identify the mutated genes.

The mutation in *E. coli* strain BMS67C12 was located in a gene previously identified as *msbB*. Karow et al., J. Bacteriol., 174:702-710 (1992) incorporated herein by reference. The *msbB* gene is found at 40.5 min. on the *E. coli* genome and the function of its product had not been identified. The *msbB* gene was originally cloned and identified due to its ability to act as a multicopy extragenic suppressor of a mutation in the *htrB* gene. Karow et al., ibid., 174:702-710 (1992); Karow et al., J. Bacteriol. 173:741-750 (1991). Part of the phenotype of the *htrB* gene, and the reason it was originally isolated, is that it is temperature sensitive and will not grow above 32°C. Karow and Georgopolis, Molec. Microbiol., 5:2285-2292 (1991). The Tn5 insertion in BMS67C12 is located immediately following the glycine codon at amino acid position number 198. Since the product of the *msbB* gene clearly plays a key role in lipid A biosynthesis the *msbB* locus is alternatively designated herein as *lpxF*, the next available genetic designation for lipid A biosynthesis (Raetz, J. Bacteriol., 175:5745-5753 (1993)).

Example 6

This Example describes the restoration of the E-selectin stimulatory LPS phenotype to BMS67C12 by the cloned *lpxF* gene.

The previously published sequence of *msbB* (Karow et al., J. Bacteriol., 174:702-710 (1992) was used to design the oligonucleotide primers 5'-TCGATCGGATCCCCACATCCGGCCTACAGTTCAATG-3' [SEQ ID NO:2] and 5'-TCGTCGCGAATTCCTGGCG-3' [SEQ ID NO:3] at the 5' and 3' ends of the *lpxF* gene. Cloning of the intact *lpxF* gene using *E.*

coli JM83 total DNA as a template was accomplished using the primers in a polymerase chain reaction (PCR) with an annealing temperature of 50°C and 2.5mM $MgCl_2$. The isolated *lpxF* gene, cloned into a pUC18 vector to form plasmid pBMS66, was then transformed into BMS67C12. Several clonal isolates were grown to late log phase at 37°C, their density adjusted to A_{660} = 0.25, and screened for the ability to stimulate E-selectin expression on HUVEC cells. Each of six clones tested displayed the same ability to complement BMS67C12, restoring a stimulatory phenotype to the cells.

The specificity of the lpxF phenotype to exclude just the myristoyl group but not the lauroyl fatty acid on the LPS, indicates that two separate enzymes may be involved in the addition of myristoyl and lauroyl fatty acids to the final lipid A structure. As seen in Table 2, whole cell fatty acid (WC) and phospholipid (PL) profiles did not vary significantly among the strains. Values in Table 2 are given as micrograms fatty acid/ milligram dry cell weight. The analysis was repeated three times with the other two analyses yielding similar results. ND = Not detected. In addition to fatty acid analysis in Table 2, gas chromatograph (GC) analysis revealed no difference between core oligosaccharide composition between JM83 and BMS67C12. Also, the phosphate composition was found to be unchanged.

TABLE 2. Fatty Acid Composition of *E. coli* strains.

Strain	Cellular Component	Fatty Acid Content						
		12:0	14:0	H14:0	16:1	16:0	18:0	18:0
JM83	LPS	2.2	2.2	6.2	ND	0.33	ND	ND
	WC	1.7	3.0	6.8	10.0	23.0	4.5	.55
	PL	.09	.93	ND	8.7	17.0	3.5	.40
BMS67C12	LPS	2.0	0.3	7.2	ND	0.35	ND	ND
	WC	1.4	1.3	6.0	11.0	25.0	4.1	0.63
	PL	0.1	1.3	ND	8.7	17.0	2.5	0.36
BMS67C12 (pBMS66)	LPS	2.6	2.6	8.7	ND	0.39	ND	ND
	WC	1.6	2.3	7.0	10.0	27.0	3.5	0.66
	PL	.07	.84	ND	8.5	16.0	3.2	0.44

Example 7

This Example demonstrates that the growth rates and susceptibility to selected detergents and antibiotics for the *lpxF* mutant and its parental strain are the same.

No differences were observed in the growth rate of the *lpxF* mutant when compared to the parent strain JM83 at 30°C or 37°C, similar to a report by Karow et al., J. Bacteriol., 174:702-710 (1992) for the *msbB* gene mutant. Karow et al. reported a four-fold difference in resistance to the detergent deoxycholate, and a mutation in the *msbB* gene (which is now known to code for the first committed step in lipid A biosynthesis) has also been found to increase susceptibility to several antibiotics, including rifampin, novobiocin and chloramphenicol. Vuorio and Vaara, Antimicrob. Agents Chemother., 36:826-829 (1992).

Example 8

This Example demonstrates the antagonistic effects of dmLPS.

In a study designed to assay for inhibition of E-selectin expression, HUVEC's (2 X 10^5/ml) were added and incubated with (+) and without (-) 10 ng/ml JM83 *E. coli* LPS. Detection of E. selectin was performed as described in Darveau et al., supra. As seen in Table 3, the data indicate that the level of stimulation by JM83 LPS could be reduced to the level of stimulation found for dmLPS alone when provided at a ratio of between 10 and 100 (dmLPS:LPS).

TABLE 3. dmLPS Inhibition of E-selectin Expression by JM83 LPS

dmLPS (ng/ml)	E-selectin (A_{450}/A_{630}) -LPS	+LPS	Std. Dev. -LPS	+LPS
10,000	0.136	0.123	0.005	0.002
1,000	0.132	0.138	0.006	0.007
100	0.151	0.433	0.009	0.013
10	0.101	0.589	0.010	0.011
1	0.052	0.588	0.005	0.019
0.1	0.053	0.591	0.010	0.006
0.01	0.053	0.606	0.004	0.016
0	0.060	0.594	0.001	0.015

Additional studies were performed using smooth LPS isolated from two *E. coli* clinical isolates (0111B4 and A016, Seattle) with results identical to that for JM83. Thus, the data demonstrate that dmLPS effectively inhibited the specific activation of endothelial cells to induce E-selectin expression.

In a related study, the ability of dmLPS to block production of TNF-α was assessed. A constant amount of JM83 LPS (10 pg/ml) was mixed with various concentrations of dmLPS and assayed for stimulatory ability by measuring TNF-α production. Various concentrations range of dmLPS alone was also assayed. Adherent monocytes were isolated and plated in a 48 well tissue culture plate as discussed above. LPS' were diluted and mixed in media at 2X concentration. The LPS mixtures were then added to an equal volume of media containing 10% normal human serum. Following mixing, 100 μl of each mixture was aliquoted onto the adherent monocytes and the cells were incubated for four hours at 37°C with 6% CO_2. Following incubation, the culture supernatants were harvested and aliquots were frozen and stored at -70°C until assayed. TNF-α concentrations were determined using a commercial assay (Amersham Corp., Arlington Heights, IL). The results of Table 4 indicate that TNF-α production by monocytes is blocked by dmLPS.

TABLE 4. TNF-α Production by Monocytes Blocked with dmLPS

dmLPS (ng/ml)	TNF-α (pg/ml) JM83 LPS	dmLPS Only
0	930	0
0.01	680	33
0.1	345	40
1	160	41
10	255	84
100		297

All publications and patents mentioned in this specification are herein incorporated by reference into the specification to the same extent as if each individual publication or patent was specifically and individually indicated to be incorporated herein by reference.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be obvious that certain changes and modifications may be practiced within the scope of the appended claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
(A) NAME: Bristol-Myers Squibb Company
(B) STREET: 345 Park Avenue
(C) CITY: New York
(D) STATE: New York
(E) COUNTRY: United States of America
(F) POSTAL CODE (ZIP): 10154
(G) TELEPHONE:
(H) TELEFAX:
(I) TELEX:

(ii) TITLE OF INVENTION: DE-MYRISTOLATED LIPOPOLYSACCHARIDE OF GRAM-NEGATIVE BACTERIA

(iii) NUMBER OF SEQUENCES: 3

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(v) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER: WO
(B) FILING DATE: 09-JAN-1997
(C) CLASSIFICATION:

(vi) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: US 60/010,089
(B) FILING DATE: 09-JAN-1996

(vii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Parmelee, Steven W.
(B) REGISTRATION NUMBER: 31,990
(C) REFERENCE/DOCKET NUMBER: 9197F-007700PC

(vii) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: 206-467-9600
(B) TELEFAX: 206-576-0300

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

ATGGAAGTCA GATCCTGG 18

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 36 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

TCGATCGGAT CCCCACATCC GGCCTACAGT TCAATG 36

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

TCGTCGCGAA TTCCTGGCG 19

WHAT IS CLAIMED IS:

1. A method for producing a heterologous protein in gram-negative bacteria, comprising:

culturing the bacteria which express a gene coding for the heterologous protein, wherein the synthesis or activity of *lpxF* of the bacteria is inhibited.

2. The method of claim 1, wherein *lpxF* genes of the gram-negative bacteria are at least 65% homologous to *E. coli lpxF* or a fragment thereof of at least 25 nucleotides in length.

3. The method of claim 1, wherein synthesis is inhibited by preventing transcription or translation of a *lpxF* gene.

4. The method of claim 3, wherein the *lpxF* is inhibited by a promoter, operator or structural gene mutation.

5. The method of claim 1, wherein the gram-negative bacteria is *E. coli*.

6. The method of claim 1, wherein the heterologous protein is a viral, bacterial or eukaryotic protein.

7. A method for producing a de-myristolated gram negative bacterial composition, comprising culturing gram-negative bacteria in which synthesis or activity of *lpxF* is inhibited in said bacteria and suspending the bacteria or products thereof in a pharmaceutically acceptable carrier.

8. The method of claim 7, wherein said gram-negative bacteria are *Salmonella typhimurium*, *Vibrio cholerae*, *Bordetella pertussis*, and *Haemophilus influenzae*.

9. The method of claim 7, wherein the product is a protein or lipopolysaccharide.

10. The method of claim 9, wherein the protein is a secreted or outer membrane protein.

11. The method of claim 8, wherein the bacteria are used as a cellular vaccine.

12. The method of claim 9, wherein the protein is a eukaryotic or viral protein.

13. A composition which comprises a heterologous gene product expressed by a gram-negative bacterium, wherein synthesis or activity of *lpxF* of the gram negative bacterium is inhibited.

14. The composition of claim 13, wherein said gram-negative bacterium is *E. coli*.

15. The composition of claim 13, wherein said gene product is an antibody or binding fragment thereof.

16. A method for antagonizing LPS-mediated activation of mammalian cells, comprising:

contacting said cells with a sufficient quantity of dmLPS such that the cells are not substantially activated by LPS.

17. The method of claim 16, wherein antagonizing LPS-mediated activation is by binding of LPS to a LPS specific receptor.

18. The method of claim 17, wherein the receptor is lipopolysaccharide binding protein or CD14.

19. The method of claim 7, wherein the product is dmLPS.

20. The method of claim 19, wherein the gram negative bacteria is *E. coli*.

21. A composition made according to the method of claim 7.

22. The method of claim 7, wherein the gram-negative bacteria is *E. coli*.

23. An LPS composition from a gram-negative bacteria with a lipid A component, comprising: β(1,6) D-glucosamine disaccharide 1, 4' bisphosphate with the following lipid placement: 2-[14:0 (3-OH)], 3-[14:0 (3-OH)], 2'-[14:0 (3-O-12:0)], 3'-[14:0 (3-OH)].

24. The LPS composition of claim 23, consisting essentially of β(1,6) D-glucosamine disaccharide 1, 4' bisphosphate with the following lipid placement: 2-[14:0 (3-OH)], 3-[14:0 (3-OH)], 2'-[14:0 (3-O-12:0)], 3'-[14:0 (3-OH)].

25. The LPS composition of claim 23 in a pharmaceutically acceptable carrier.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/00392

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :Please See Extra Sheet.
US CL :Please See Extra Sheet.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/69.1, 69.1, 71.1; 514/2; 424/203.1, 240.1, 256.1, 257.1, 261.1.

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Dialog

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	SOMERVILLE JR. et al. A Novel Escherichia coli Lipid A Mutant That Produces an Antiinflammatory Lipopolysaccharide. Journal of Clinical Investigation, January 1996, Volume 97, Number 2, pages 359-365, see pages 359, 361, 363.	1-20

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
27 APRIL 1997	22 MAY 1997
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer H. F. SIDBERRY Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/00392

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (6):

A61K 39/10, 39/018, 39/102, 39/108, 39/106, 39/116; A01N 37/18; C12P 21/06

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

435/69.1, 69.1, 71.1; 514/2; 424/203.1, 240.1, 256.1, 257.1, 261.1.

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

Group I, claim(s) 1-6, 13-15, drawn to method of producing a heterologous protein.
Group II, claim(s) 7-12, 19-22, drawn to method for producing a demyristolated gram-negative bacteria.
Group III, claim(s) 16-18, drawn to method for antagnizing LPS mediated activity of mammalian cells.
Group IV, claim(s) 23-25, drawn to LPS composition.